FEATURES OF MULTIPLICATION OF DIFFERENT CLONES OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS IN AEDES AEGYPTI MOSQUITOES

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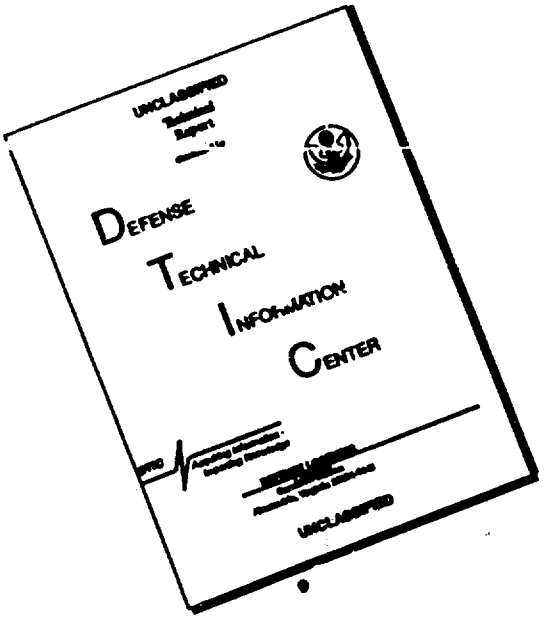

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Features of multiplication of different clones of Venezuelan equine encephalomyelitis virus in Aedes aegypti mosquitoes

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Many data have recently accumulated concerning the fact that "wild" populations of arboviruses, circulating in nature, are very inhomogeneous in the genetic sense. An active link in the natural cycle of arboviruses are bloodsucking arthropods, therefore it is fully probable that they can exert a substantial influence upon the composition of the "wild" population, selectively picking those variants which multiply most actively in the carrier. The essence of these phenomena remains undisclosed. In the present stage of the development of science, it has become possible to study this process experimentally with the use of genetically stable clones of arboviruses. In our experiments, a comparative study was made of the multiplication of different clones of Venezuelan equine encephalomyelitis virus (VEE) in Aedes aegypti mosquitoes, which differ from each other by the degree of pathogenicity for white mice and by virtue of some other genetic characteristics.

Materials and Methods

Used in the project was a "wild" strain of VEE virus, and clones 3/5 and 17 isolated from it (Table 1), as well as 2 mutants induces by low temperature, avirulent for white mice, clones 53 and 56. Clones 3/5 and 5 were characterized by high virulence and constituted variants predominating in the population of the "wild" virus, while clone 17 was among those contained in a natural population of "spontaneous" mutants with lowered pathogenicity for mice.

The culture of Aedes aegypti was obtained from the Institute of Medical Parasitology and Tropical Medicine imeni Ye. I. Martsinovskiy of the Ministry of Public Health, USSR. The mosquitoes were maintained at a temperature of 27° and a relative humidity of 80-90%. Their infection by the virus was caused by their feeding on infected mice or through an animal membrane - on the skins of mouse tails filled with a mixture of virus with the defibrinated blood of a rabbit or a mouse in the ratio of 1:1. The virus contain in the suspension comprised 10^6 - 10^8 conventional biological units /?/ (CBU) in 0.2 ml.

Table 1. Genetic criteria of clones of VEE virus.

a) Клон	b) Происхождение	c) Генетические признаки			
		i/c	i/p	S	T_{60}
3/5	Естественный e)	7,6	7,65	8,31±0,21	10
5	»	7,2	7,1	3,65±0,03	3
17	«Спонтанный» мутант	5,3	3,2	1,72±0,03	1
53	Индуцированный мутант f)	0,5	0,5	3,56±0,09	5
56	» »	0,5	0,5	0,73±0,02	4

Key to Table 1. a) clone; b) origin; c) genetic characteristics e) "spontaneous" mutant; f) induced mutant

Designations: i/c and i/p - pathogenicity for white mice during intracerebral and intraperitoneal infection. The value of $lgLD_{50}$(?, subscript illegible)(0.2 ml when titrating for mouse viruses containing from 2 to 8 x 10^6 CBU in 0.2 ml). S is the plaque size (in mm). . The average diameter and the mean error are given; T_{60} is the resistence to warming at 60°. The total inactivation time of 5 x 10^5 CBU of the virus is given in minutes.

For the determination of viremia, the blood from 4-5 mice or 1-2 hamsters, or guinea pigs, was collected in a test tube with gemarin, the prescribed elements were precipitated, and virus was titrated in the fluid above the precipitate by the plaque method. The accumulation and dynamics of multiplication of the virus in mosquitoes was studied, as well as the presence of virus

in the mucous glands and its transmission via bite.

Quantitative determination of the virus in mosquitoes was conducted by titrating a suspension from mosquito bodies. In each test tube, 8-15 specimens were combined. After rinsing in physiolcgical solution with antibiotics, they were transferred into a crude porcelain beaker and were crushed to a homogeneous mass, then physiological solution was added on the basis of 0.1 ml per mosquito. After centrifuging for 15 minutes at 2000 RPM, the liquid above the precipitate was used for titration by the plaque method , considering this suspension to be the initial undiluted material.

The presence of a viral antigen in salivary glands was determined by the method of immunofluorescence. Ascitic fluid immune to the VEE virus, conjugated with fluorescin thiocyanate, was used as the labelled antibodies. The virus antigen was isolated by means of the direct method of fluorescence against the contrasting background of nonspecific luminescence by beef albumin, labelled by rhodamine sulfofluoride. Staining the mucous glands was based on the method used by Takahaci of the National Institute of Public Health in Tokyo. The isolated mucous glands were fixed on a slide in acetone for 10 minutes, were dried, stained by labelled serum, and were contrasted by rhodamine-labelled albumin. During staining and contrasting, the preparation, together with the applied mixture of conjugated globulin and albumin, was carefully covered by a cover glass, was placed in a moist chamber, and was left 18 hours for contact at a temperature of 4°. Thereafter, without removing the cover glass, the preparations were thoroughly rinsed by a phosphate buffer solution with pH 7.2 by means of undercoating and suction of the liquid. After rinsing, the preparations were ready to be studied in a luminescent microscope at a magnification of 10 x 40.

Results

In experiments on the infection of mosquitoes by feeding on infected mice, it was established that not each of the studied viruses is transmitted by arthropods from infected animals. Whereas after feeding on mice infected by an uncloned "wild" virus and highly virulent clones 5 and 3/5, the virus was isolated regularly from the carriers, avirulent clones 53 and 56, as well as weakened clone 17, could not be isolated from mosquitoes (Table 2).

Since weakened and avirulent variants of the virus, which were not transmitted from infected mice by the carriers, caused low viremia in white mice (see Table 2), as well as in young hamsters, guinea pigs, and rabbits, it was of interest to determine the minimal content of the virus in mouse blood, at which infection of the carriers still takes place. Such a threshold value of viremia for clone 355 was 2 x 10^3 CEU/0.2 ml, for clone 5 it was 6 x 10^3 CEU/0.2 ml, and for the uncloned virus it was 9 x 10^3 CEU/0.2 ml. The maximum value of viremia caused by avirulent clones 53 and 56, as well as by weakened clone 17, did not reach this threshold.

Table 2. Isolation of the VEE virus from Aedes aegypti mosquitoes after feeding on mice infected by different virus variants

Вирус	Титр вируса в крови мышей (доза в КОЕ/0.2 мл)	Наличие вируса в комарах: время после кормления (в неделях)			
		1	2	3	4
Неклонированный	$3 \cdot 10^5$	+	+	+	—
Клоны:					
3/5	$4 \cdot 10^6$	—	—	+	+
5	$2 \cdot 10^5$	+	+	+	—
17	$8 \cdot 10^1$	—	—	—	Кормление не проводилось
53	$5 \cdot 10^1$	—	—	—	
56	$3 \cdot 10^1$	—	—	—	—

Key to Table 2: a) virus; b) uncloned; c) clones:; d) viremia in donor mice, CBU/0.2 ml; e) presence of virus in mosquitoes; f) time after feeding (in weeks); g) no feeding was conducted

Unsuccessful attempts to isolate the virus from mosquitoes after feeding on mice infected by avirulent and weakened virus were not linked to the fact that these variants cannot multiply in carriers at all, but were caused merely by the low content of virus in the blood of the donor mice and, consequently, by an insufficient infective dose. When the mice were fed by a virus suspension through an animal membrane, infection of the carriers and multiplication of the virus was observed independently of the genetic properties of the virus. Moreover, a virulent (uncloned virus, clones 3/5 and 5) and an avirulent virus (clones 53 and 56) were accumulated in the mosquito organism in various quantities. A weakened virus (clone 17) multiplied intensively in mosquitoes (its content reached titers that are characteristic of the virulent variants (see figure).

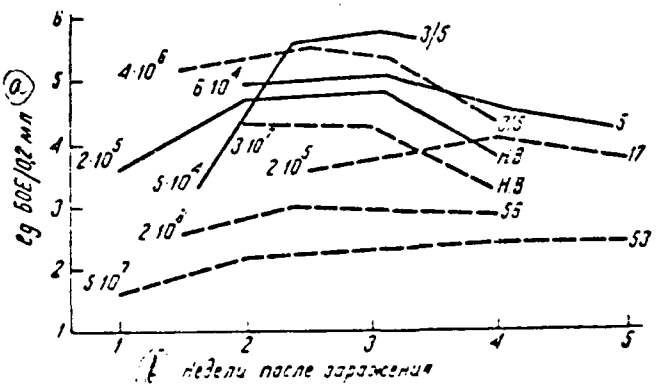

Figure: accumulation of VEE virus in Aedes aegypti mosquitoes.
solid line - feeding on infected mice;
dotted line - through membrane.
4.10^6 - viremia in donor mice or virus content in the suspension fed to the mosquitoes, in CBU/0.2 ml; 3/5, 5, 17, 53, and 56 - clone number; H. B. - uncloned virus.

Key to figure: a) lg CBU/0.2 ml; b) weeks after infection

Not only quantitative, but also qualitative differences between natural virus variants, existing in nature, and those obtained in the laboratory, were observed during multiplication in mosquitoes. Although the modified virus (clones 53 and 56) did multiply in mosquitoes, it did not penetrate into the mucous glands (virus antigen was not detected in mucous glands by the immunofluorescence method). In biological experiments the virus was not transmitted by bite (Table 3). In experiments with natural variants of the virus (clones 17, 3/5, 5, and the uncloned virus), a specific virus antigen was detected in the mucous glands; this testifies to the penetration of virus into the mucous glands and its multiplication in the cells of this organ. The luminescence of the antigen accumulating in the cells of the mucous glands was of a bright yellow-green color. Control glands and gland sectors of infected mosquitoes, not containing the virus antigen, had an intense red-orange color. The infected mosquitoes transmitted the infection to mice by bite (see Table 3).

Table 3. Capability of natural and modified variants of VEE virus for multiplying in the mucous glands and being transmitted by the bite of infected Aedes aegypti mosquitoes

(a) Вирус	Размножение вируса в комарах	Наличие вирусного антигена в слюнных железах	Передача через укус
(b) Неклонированный	+	+	+
Клоны:			
(c) 3/5	+	+	+
5	+	+	+
17	+	+	+
56	+	—	—
53	+	—	—

Key to Table 3: a) virus; b) uncloned; c) clones:; d) multiplication of virus in mosquitoes[1]; e) presence of virus antigen in mucous glands[1]; f) transmission by bite[1]

Footnote[1]: Determined periodically within a period from one to eight weeks after infection of mosquitoes.

Designations: + all tests positive; - negative.

Discussion

We conducted the experiments on laboratory animals that are not natural hosts of the virus, and on Aedes aegypti mosquitoes, which are not included among the carriers of the virus in the principal foci of infection. Nevertheless, the attained experimental data make it possible to express some suppositions concerning the factors which exert an influence upon the genetic composition of the VEE population circulating in nature.

High-virulent variants of the virus, which in our experiments were the uncloned virus and clones 3/5 and 5, caused high viremia, sufficient for infection of the carriers; they penetrated into the mucous glands and were transmitted by bite. The circulation cycle of the virus in the studied experimental system was completed; a continuous cycle of the causative agent was provided for. Modified variants, avirulent for white mice (clones 53 and 56), did not possess the capacity for circulation, since they caused a viremia that was too low for infecting the carriers. With experimental infection of the carriers, these variants of the virus did not accumulate in the mucous glands and were not transmitted by bite. Although a weakened variant of the virus (clone 17) was transmitted by bite, it was also incapable of circulation, since it caused a virus that was insufficiently intense for infecting the carriers. The incapacity of this weakened virus variant for circulation evokes particular interest, since it is not an induced virus, obtained in the laboratory, but is a "spontaneous"

mutant present in the virus population. It is entirely probable that when such variants appear in the primary hosts or in the carriers under natural conditions during multiplication of the virus, they can not be transmitted to the mosquitoes and are therefore incapable of displacing the predominant genotypes.

Thus, it can be supposed that circulation of the weakened variants of VEE virus under natural conditions is interrupted at the stage of transmission from the primary host to the carrier, while circulation of the mutants that are avirulent to white mice is interrupted both at the stage of transmission to the carrier and at the stage of transmission from the carrier. By virtue of the biological advantages noted here, the pathogenic virus is transmitted from the primary hosts to the carriers and conversely, completing the circulation cycle without hindrance. The obtained data make it possible to explain the fact that although VEE virus strains that are isolated in nature do vary antigenetically, with respect to plaque size, and with respect to some other characteristics they do not differ essentially with respect to the degree of pathogenicity and belong to the "wild" highly virulent variants of the causative agent. Naturally attenuated variants of the VEE virus were not discovered in natural infection foci.

Summary

Multiplication in *Aëdes aegypti* mosquitoes of clones of Venezuelan equine encephalomyelitis virus (VEE) with varying pathogenicity for white mice, as well as their capacity to accumulate in salivary glands and be transmitted by bite were studied comparatively. Natural virulent variants of VEE virus, in contrast to induced mutants avirulent for white mice, multiplied intensively in *Aëdes aegypti* mosquitoes, accumulated in salivary glands and were transmitted by bite. The distinctive property of virulent variants consisted also in their capacity to cause intensive viremia. The experimental evidence suggests that these biological properties of virulent variants may ensure their permanent circulation in nature and prevalence in the natural population of VEE virus. Induced mutants avirulent for white mice are not adapted to existence under natural conditions since during multiplication in vectors they do not penetrate into salivary glands and are not transmitted by bite, and during multiplication in mice their amount in the blood is not sufficient for infection of vectors. Low viremia also limits circulation of "spontaneous" mutants with low pathogenicity.